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GAU-1614
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Practitioner's Docket No. 49592 (1878)

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: D. Williams et al.
Serial No.: 09/506,362 Group No.: 1614
Filed: February 15, 2000
For: APOPTOSIS-INDUCING COMPOUNDS

Assistant Commissioner for Patents
Washington, D.C. 20231

TRANSMITTAL OF CERTIFIED COPY

Attached please find the certified copy of the foreign application from which priority is claimed for this case:

Country: Ireland
Application Number: 980344
Filing Date: 6 May 1998

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SIGNATURE OF PRACTITIONER

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NOTE: "The claim to priority need be in no special form and may be made by the attorney or agent, if the foreign application is referred to in the oath or declaration, as required by § 1.63." 37 C.F.R. 1.55(a).

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Susan M. Dillon
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I HEREBY CERTIFY that annexed hereto is a true copy of documents filed in connection with the following patent application:


Application No. 980344

Date of Filing 6 May, 1998

Applicant THE PROVOST, FELLOWS AND SCHOLARS
OF THE COLLEGE OF THE HOLY AND
UNDIVIDED TRINITY OF QUEEN ELIZABETH
NEAR DUBLIN, a Body Corporate under Irish Law
of Trinity College, Dublin University, Dublin 2,
Republic of Ireland.

Dated this 10, day of May, 2000.




An officer authorised by the
Controller of Patents, Designs and Trademarks.
PP

REQUEST FOR THE GRANT OF A PATENT

PATENTS ACT, 1992

The Applicant(s) named herein hereby request(s)

☒ the grant of a patent under Part II of the Act

☐ the grant of a short-term patent under Part III of the Act
on the basis of the information furnished hereunder.

1. Applicant(s)

Name THE PROVOST, FELLOWS AND SCHOLARS OF THE COLLEGE OF THE HOLY AND UNDIVIDED
TRINITY OF QUEEN ELIZABETH NEAR DUBLIN

Address Trinity College, Dublin University, Dublin 2, Republic of Ireland

Description/Nationality a Body Corporate under Irish Law

2. Title of Invention

"APOPTOSIS - INDUCING COMPOUNDS"

3. Declaration of Priority on basis of previously filed
application(s) for same invention (Sections 25 & 26)

<u>Previous filing date</u>	<u>Country in or for which filed</u>	<u>Filing No.</u>
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one

4. Identification of Inventor(s)

Name(s) of person(s) believed
by Applicant(s) to be the inventor(s)

Address DAVID CLIVE WILLIAMS
24 Dalkey Park, Dalkey, Co. Dublin,
Ireland

DANIELA M. ZISTERER
49B York Roads, Dun Laoghaire, Co. Dublin,
Ireland

5. Statement of right to be granted a patent (Section 17(2)(b))
by virtue of an Assignment dated

6. Items accompanying this Request - tick as appropriate

- (i) ☒ prescribed filing fee (£ 100)
- (ii) ☒ specification containing a description and claims
☐ specification containing a description only
☒ Drawings referred to in description or claims
- (iii) ☐ An abstract
- (iv) ☐ Copy of previous application(s) whose priority is claimed
- (v) ☐ Translation of previous application whose priority is claimed
- (vi) ☒ Authorisation of Agent (this may be given at 8 below if this Request is signed by the Applicant(s))

7. Divisional Application(s)

The following information is applicable to the present application which is made under Section 24 -

Earlier Application No:
Filing Date:

8. Agent

The following is authorised to act as agent in all proceedings connected with the obtaining of a patent to which this request relates and in relation to any patent granted -

Name

Address

TOMKINS & CO.

5, Dartmouth Road,
Dublin 6.

9. Address for Service (if different from that at 8)

TOMKINS & CO., at their address as recorded for the time being in the Register of Patent Agents.

TOMKINS & CO., Authorised Patent Agents.

Signed

Name(s): by: *M. C. Gyles*
Capacity (if applicant is a body corporate):

Date 6 May 1998

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Apoptosis-Inducing Compounds

- 1 -

ADMINISTRATIVE

The present invention relates to pyrrolobenzoxazepine compounds having the ability to induce apoptosis, to pharmaceutical compositions comprising these compounds and to their use as anti-tumour agents.

Background to the Invention

Benzodiazepines such as Valium are amongst the most highly prescribed drugs due to their anxiolytic, relaxant and sedative effects. Target-specific binding of benzodiazepines has been observed in many tissues and cell types and can be separated pharmacologically into two classes (see Table 1). The first and more widely studied of the two are the "central-type" binding sites located in brain which mediate the clinical effects of the benzodiazepines. These "central-type" sites are associated with the neuronal postsynaptic gamma-aminobutyric acid receptor which regulates Cl^- flux (Costa and Guidotti, 1979). Benzodiazepines also bind to another distinct binding site (Braestrup and Squires, 1977; Schoemaker et al., 1981) found in the membranes of many mammalian tissues: heart, kidneys, lungs, adrenals, blood platelets and also the brain, termed the peripheral-type benzodiazepine receptor (PBR).

Description of the Prior Art

The exact function of the PBR is unclear although PBR specific ligands such as Ro5-4864 (4'-chlorodiazepam) and PK11195 (1-(2-chlorophenyl)-1,3-dihydro-1-methyl-propyl) isoquinoline carboxamide) have been shown to elicit a wide variety of effects including alteration in cardiac action potentials and calcium channels, alterations of proto-oncogene expression modulation of steroidogenesis and alteration of immune function (Zisterer & Williams, 1997 for review). There have been many reports that benzodiazepines affect cell growth and differentiation in a number of cell types. These include induction of differentiation of Friend erythroleukemia cells (Wang et al., 1984a) and inhibition of cell proliferation (Wang et al., 1984b; Gorman et al., 1989; Ikezaki and Black, 1990; Camins et al., 1995). However, because of the high concentrations of PBR ligands necessary to elicit these

antiproliferative effects and the demonstration of similar effects in cell lines which lack the PBR (Gorman *et al.*, 1989), it could be concluded that these antiproliferative effects seem unrelated to a specific interaction of these drugs with this receptor.

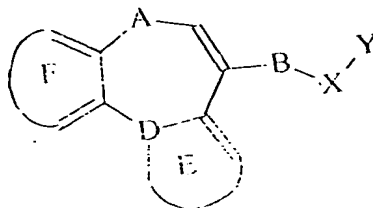
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Recently a novel series of high affinity PBR ligands based on a pyrrolobenzoxazepine skeleton, classified here as NF compounds, have been synthesised (Campiani *et al.*, 1996). A recent study (Zisterer *et al.*, 1998) has demonstrated that three of these novel PBR ligands, NF 182, 213 and 262 along with the classically used PBR ligands PK 11195 and Ro5-4864 were found to inhibit at micromolar concentrations and in a dose-dependent manner, the proliferation of rat C6 glioma and human 132 1 N1 astrocytoma, without being cytotoxic. This antiproliferative effect was found to be mediated by arrest in the G1 phase of the cell cycle.

15

The present inventors have, while examining the effect of PK 11195, Ro5-4864 and the NF compounds on the proliferation of the human cancer cell lines, Jurkat (leukaemic T cell lymphoblast), HL-60 (promyelocytic leukaemia) HUT 78 (T cell lymphoma) and LAMA, KYO.1 and 20 K562 cells which are all CML (chronic myeloid lymphoma) cells, determined that these PBR ligands induce apoptosis, with various potencies, in the six cell lines. Apoptosis is a cell suicide mechanism invoked in disparate situations, both physiological and pathological, to ablate unwanted, damaged, or potentially neoplastic cells. Apoptosis is 25 classically defined by a characteristic set of morphological changes in the cell, including membrane blebbing, cell shrinkage, chromatin condensation, DNA fragmentation and eventual formation of membrane-bound apoptotic bodies. Although a number of reports have identified agents that are capable of inducing apoptosis in a variety of cells and 30 tissues, the mechanisms responsible for the activation of the apoptosis process are poorly understood.

According to the present invention there is provided a pharmaceutical composition comprising an apoptosis-inducing amount of a 35 compound having the general formula:-



wherein R_1 represents

unsubstituted straight chain, or branched C_1-C_{10} alkyl or
unsubstituted C_3-C_{10} cycloalkyl,

5 or straight chain or branched C_1-C_{10} alkyl substituted with
one or more substituents or C_3-C_{10} cycloalkyl substituted with one
or more substituents,

unsubstituted straight chain, or branched C_2-C_{10} alkylene or
unsubstituted C_3-C_{10} cycloalkylene,

10 or straight chain or branched C_2-C_{10} alkylene substituted with
one or more substituents or C_2-C_{10} cycloalkylene substituted with
one or more substituents,

an unsubstituted phenyl group or phenyl substituted by one or more
15 substituents,

an unsubstituted C_6-C_{20} aryl group or a C_2-C_{20} aryl group
substituted with one or more substituents,

unsubstituted naphthyl substituted with one or more substituents,
preferably the substituted or unsubstituted naphthyl being a 1- or 2-
20 naphthyl,

an unsubstituted biphenyl or a biphenyl substituted with one or
more substituents, preferably the substituted or unsubstituted biphenyl
being a 4- biphenyl,

25 an unsubstituted five or six numbered heterocyclic group with at
least one hetero atom and wherein the or each heteroatom is selected
from N,O,S or C, five or six numbered heterocyclic group with at least
one heteroatom and wherein the or each heteroatom is selected from
30 N,O,S, the heterocyclic group being substituted with one or more
substituents preferably the heterocyclic being selected from 2- and 3-
pyridine, pyrrole, thiophene;

where A represents N,O,S or the group CH_2 ; and
35

where B represents N,O, or the group CH_2 ; and

where D represents N or the group CH_2

wherein the cyclic group labelled E is taken together with D to form a pyrrole or indole ring which may be substituted;

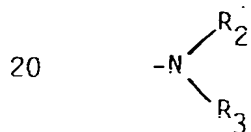
and wherein the cyclic group labelled F represents:

5 an unsubstituted phenyl group or phenyl substituted by one or more substituents,

an unsubstituted C₆-C₂₀ aryl group or a C₂-C₂₀ aryl group substituted with one or more substituents,

unsubstituted naphthyl substituted with one or more substituents,
10 or a 5 or 6 membered heterocyclic group, with at least one heteroatom and wherein the or each heteroatom is selected from O, N or S, and a 5 or 6 membered heterocyclic group with at least one heteroatom and where the or each heteroatom is selected from O, N or S substituted with one or more substituents, and preferably when the heterocycle is pyridine or
15 thiophene wherein X represents the group X=O, C=S or CH₂; and

where Y represents the group



wherein R₂ and R₃ are independently hydrogen, or

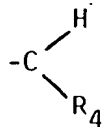
unsubstituted straight chain, or branched C₁-C₁₀ alkyl or
25 unsubstituted C₃-C₁₀ cycloalkyl,

or straight chain or branched C₁-C₁₀ alkyl substituted with one or more substituents or C₃-C₁₀ cycloalkyl substituted with one or more substituents,

unsubstituted straight chain, or branched C₂-C₁₀ alkylene or
30 unsubstituted C₃-C₁₀ cycloalkylene,

or straight chain or branched C₂-C₁₀ alkylene substituted with one or more substituents or C₂-C₁₀ cycloalkylene substituted with one or more substituents,

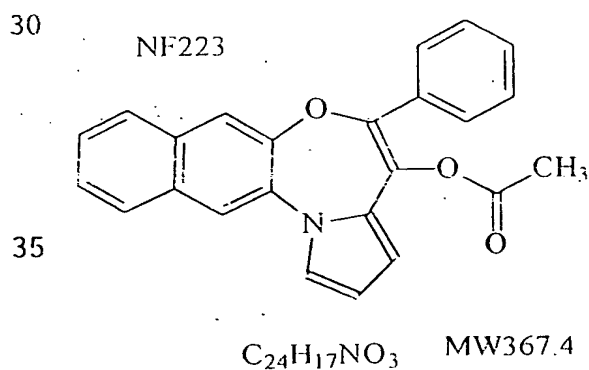
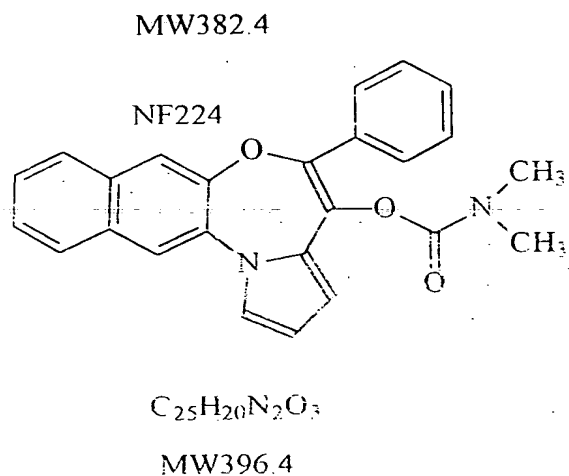
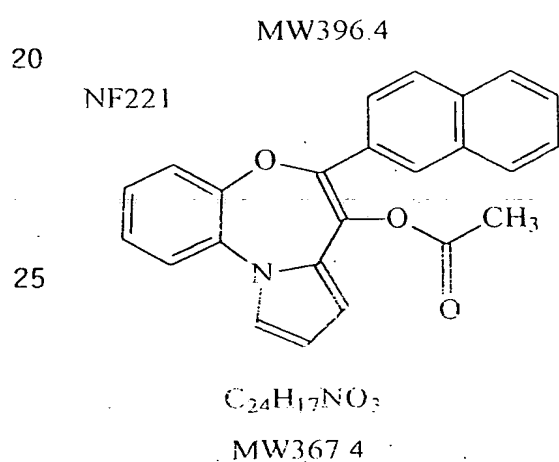
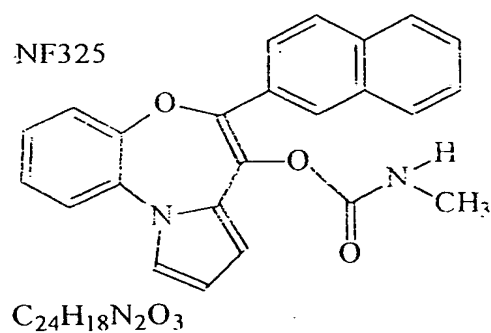
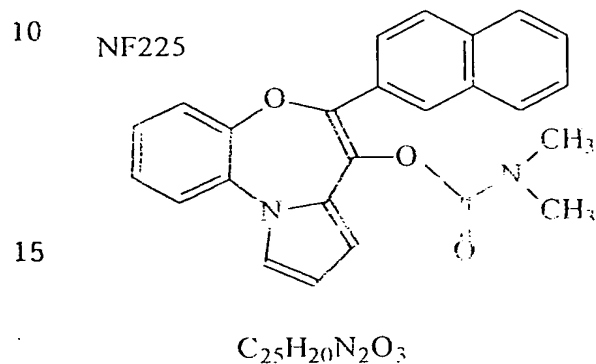
35 or Y represents the group

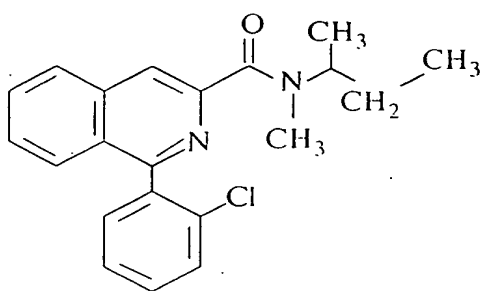


wherein R_4 is $(CH_2)_n CH_3$ wherein n is 0 or an integer from 1 to 12 and may be substituted.

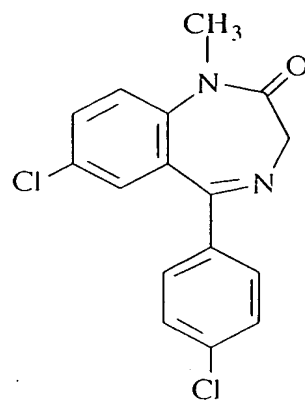
Particularly preferred are compounds in which R_1 or F represent a naphthyl group.

Most preferred are compounds selected from those having the formulae:-

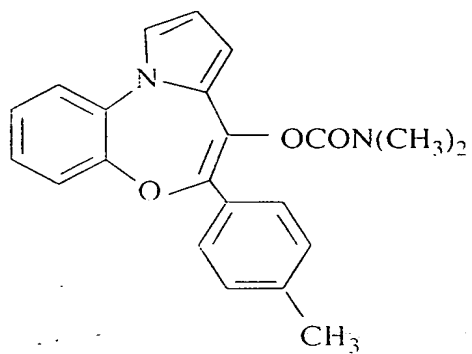




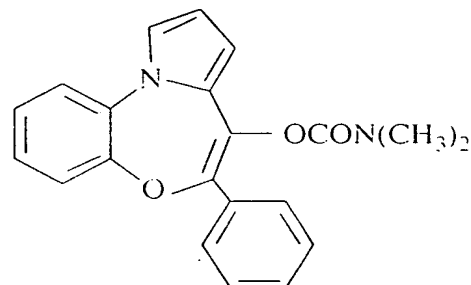
PK 11195



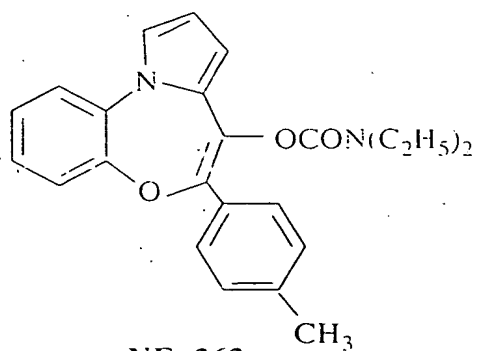
Ro5-4864



NF 213



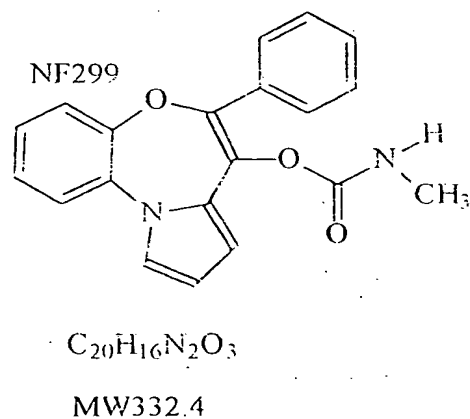
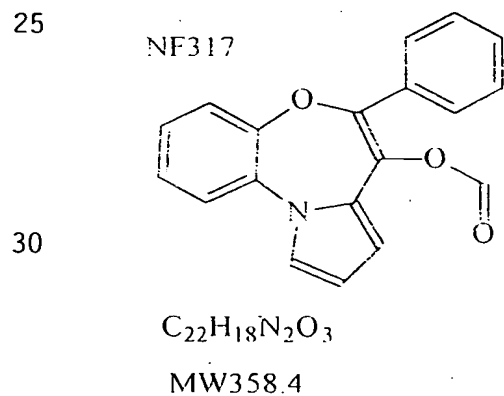
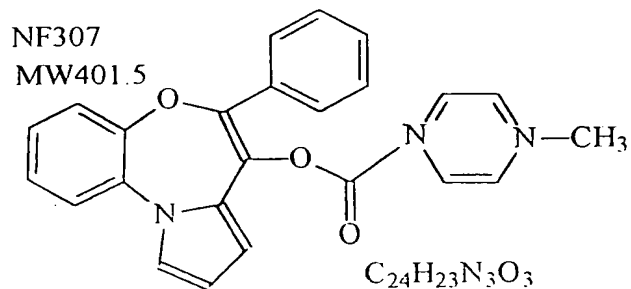
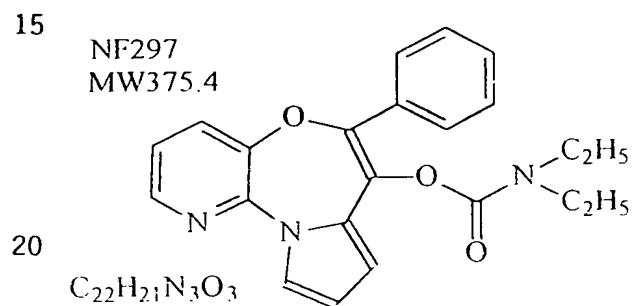
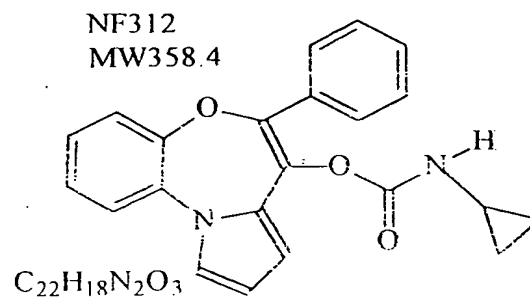
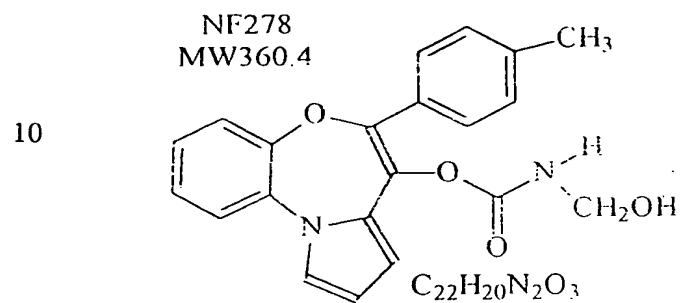
NF 182



NF 262

Other suitable compounds are PK11195 (1-(2-chlorophenyl)-1,3-dihydro-1-methyl-propyl) isoquinoline carboxamide) and Ro5-4864 (4'-chlorodiazepam).

5 Also preferred are novel compounds selected from:-



35

The invention also provides a method for the synthesis of products with the above mentioned formulae which can be performed following already known methodologies. Starting from the appropriate 2-hydroxyarylamines, the pyrrole or the indole rings are introduced by standard methods. Alkylation of the hydroxy groups with suitable aryl or alkyl acetic acid ethyl esters followed by saponification provides the corresponding acids which are cyclized under Friedel-Crafts conditions. The ketones thus obtained are functionalized prior transformation in the corresponding potassium enolates (B = oxygen). On the other hand, for products with B = CH₂, the ketones are transformed in the corresponding enol triflates which, after exposure to carbon monoxide, in the presence of Pd (0), and alcohols or amines, leads to esters and amides. Esters are reduced to alcohols, transformed to the corresponding nitriles, and transformed to the (thio)ester or (thio)amides.

The methods are described in greater detail in Campiani *et al*, 1996a and 1996b.

The invention also relates to the use of any of the above-defined compounds in the preparation of a medicament for the treatment of tumours or other cancerous conditions.

MATERIALS AND METHODS

25

Materials

HL 60 (ECACC No. 881 12501), Jurkat T (ECACC No. 88042803) and HUT 78 (ECACC No. 88041901) cells were obtained from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, U.K. The LAMA 84, KY0.1 and K562 cells were the gift of Dr. Mark Lawlor of St. James' Hospital, Dublin. The benzodiazepine, Ro 5-4864 [7chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2-H-1,4-benzodiazepin-2-one] was obtained from Fluka, Chemie AG, Buchs, Switzerland. PK-11195 [1-(2-chlorophenyl)-N-methyl-N-(1 methylpropyl)-3-isoquinolinecarboxamide] was a gift from Dr. Alan Doble, Pharmuka Laboratories, Gennevilliers, France. The pyrrolobenzoxazepine derivatives (NF compounds) were synthesized by Prof. Giuseppe Campiani, University of Siena, Italy. The RapiDiffTM kit was obtained from

Diagnostic Developments, Burscough, Lancashire, U.K. The 22 bp oligonucleotide containing the NF kappa B consensus sequence was from Promega (Madison, WI, USA). Anti-procaspase 3 was a monoclonal antibody against caspase 3/CPP32B, raised against a 24.7 kDa fragment

- 5 corresponding to amino acids 1-219 of human caspase 3 and was obtained from Transduction Laboratories (Lexington, KY, USA). z-DEVD-fmk was supplied by Calbiochem-Novabiochem, Nottingham, U.K. The enhanced chemiluminescence (ECL) kit was from Amersham Corp. (U.K.). All other reagents, including RPMI 1640, fetal calf serum, TNF- α ,
10 staurosporine and the antioxidant compounds, were supplied from Sigma (Poole, Dorset, U.K.). Diazepam and Clonazepam, N-acetyl-cysteine and butylated hydroxyanisole, TNF alpha, PDTC (pyrrolidithiocarbamate) and TEMPO (TM) were obtained from Sigma (Poole, Dorset, U.K.).

15 **Cell culture and cytospin analysis of apoptosis**

- HL 60 human promyelocytic leukaemia cells were maintained as logarithmically growing cultures in 80% RPMI 1640 medium and 20% heat-inactivated fetal calf serum (FCS) in a humidified incubator with
20 95% air, 5% CO₂ at 37°C. The HUT 78 human T cell lymphoma, and the Jurkat human leukaemic T cell lymphoblast cells and the three CML cells were grown under the same conditions as above with the exception of the FCS which was at 10%. In all experiments performed, cells were seeded at a density of 3×10^5 cells/ml into 24-well plates (1ml well) and
25 immediately treated with the indicated compound. All drugs were diluted from freshly prepared ethanol stock solutions. The final concentration of ethanol in the wells was always 1% (vol/vol); a concentration which by itself had no effect on the cells tested. Following exposure to the appropriate drug, the cells (100 μ L) were cytospun onto glass slides
30 pre-coated with poly-L-lysine using a Cytospin 3TM cytocentrifuge (Shandon). They were then stained using the Rapi-Diff kit (eosinmethylene blue) under the conditions described by the manufacturer. The percent apoptosis and necrosis was determined by counting approximately 300 cells under a light microscope with the aid
35 of a graticule inserted into the eyepiece. At least 3 fields of view per slide, with an average of approximately 100 cells per field, were counted.

Assay for NF kappa B binding activity

Cells were fractionated, and nuclear extracts were prepared as described by Stylianou *et al.* (1992). Briefly, cells were added to
5 ice-cold phosphate-buffered saline and centrifuged using a swing out rotor at 136 X g. They were washed in buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 10 mM phenylmethylsulfonyl fluoride [PMSF]) and centrifuged at 10 000 X g for 10 min. They were treated with buffer A + 0.1% (vol/vol) Nonidet P40 on ice for 10 min. They were then centrifuged
10 at 10 000 X g for 10 min and the pellet was treated with buffer C (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 2 mM EDTA, 20% [vol/vol] glycerol, 0.5 mM PMSF) on ice for 15 min. This material was centrifuged at 10 000 X g for 10 min and the supernatant was added to 75µl of buffer D [10mM Hepes, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 20% (vol/vol) glycerol,
15 0.5 mM PMSF] per sample. The protein concentration in this crude nuclear extract was determined using the method of Bradford (1976). Protein (4 µg) was incubated with 10 000 cpm of a ³²P-labelled oligonucleotide, containing the consensus sequence for NF kappa B, with binding buffer [40% (vol/vol) glycerol, 1 mM EDTA, 10 mM Tris, pH 7.5, 100mM NaCl, 0.1
20 mg/ml nuclease free BSA] and 2 µg of poly(dI-dC) at room temperature for 30 min. Samples were resolved on a 5% native polyacrylamide gel which was subsequently dried and autoradiographed at -70°C overnight.

Assay for caspase 3 using Western blotting

25

Whole cell extracts from approximately 1×10^7 cells were isolated as follows. Cells were centrifuged at 600 X g for 5 min and the resulting pellet washed in sterile PBS (1ml) and transferred into a minifuge tube. The sample was again centrifuged at 600 X g for 5 min and
30 the supernatant carefully removed. The pellet was resuspended in 0.2 ml of ice-cold RIPA [PBS, 1% (vol/vol) Nonidet P40, 0.5% (wt/vol) sodium deoxycholate, 0. 1% (wt/vol) sodium dodecyl sulphate (SDS), 100µM PMSF, 3% (vol/vol) aprotinin and 100 µM sodium orthovanadate] and incubated on ice for 30 min. The cells were then homogenised by passage through a 21G
35 needle (5-7 times) on ice. Following a further incubation on ice for 30 min, the sample was centrifuged in a minifuge at 4°C for 20 min. Supernatant (150µl) was removed into fresh minifuge tubes (whole cell lysate) and the protein concentration determined by the method of Bradford (1976). Extracts were frozen at -70°C until use. The volume of

each thawed sample was adjusted with RIPA so that equivalent amounts of protein were present in each sample. Samples were resolved on a 15 % polyacrylamide gel containing 0.1% (wt/vol) SDS and immediately transferred onto nitrocellulose membrane. The membranes were blocked overnight in PBST (PBS containing 0.05% [vol/vol] Tween-20) containing 5% (wt/vol) powdered milk (Marvel), incubated for 1 h with anti-procaspase 3 diluted (1:1000) in 5% (wt/vol) Marvel/TBST and then washed 3 times with PBST for 5 min each. This was followed by the incubation of the membranes in goat anti-mouse horseradish peroxidase-coupled secondary antibody at a dilution of 1:1000 in 5% (wt/vol) Marvel/TBST, for 45 min. Enhanced chemiluminescence (ECL) was used to visualise the cross-reacting bands, following a protocol described by the manufacturer.

15 RESULTS

Apoptosis was induced, with various potencies, by PBR ligands in HL 60 cells (Table 2). The characteristic morphological effects of apoptosis i.e. shrinkage of cells, extensive blebbing, condensation of chromatin and DNA fragmentation, were observed in treated cells. Of the PBR ligands tested (n=23), NF 221, 223, 224, 225 and 325 were the most potent inducers. Some NF compounds (e.g. NF 149, 182, 198, 212, 213 and 262) had no effect. Treatment of HL 60 cells with any one of the five most potent drugs, at a final concentration of 50 μ M (limit of solubility), resulted in between 52% (NF 221) and 67% (NF 325) apoptosis after 16 h. The degree of necrosis (percentage) observed under the same conditions was in the range of 3%-8%. Fig. 1 shows the effect of these five selected NF compounds (at a final concentration of 10 μ M) on apoptosis in HL 60 cells. Most of the subsequent experiments were performed using NF 225 as a "model" NF compound, at a final concentration of 10 μ M. Treatment of HL 60 cells with this concentration of NF 225 for 16 h, resulted in a 40% induction of apoptosis.

When HL 60 cells were incubated with NF 225, a dose- and time-dependent induction of apoptosis was observed (Fig. 2 & 3). Apoptosis of treated cells was negligible below a final concentration of 1 μ M NF 225 or for less than four hours incubation (at 10 μ M NF 225). Percent necrosis increased dramatically when cells were treated for over 16 h (data not shown).

To determine whether the PBR was involved in the mechanism by which NF compounds induce apoptosis, the effect of the five most potent NF compounds was investigated in Jurkat cells. Jurkat cells have previously been reported to lack the peripheral-type benzodiazepine receptor (Carayon *et al.*, 1996). Fig. 4 shows that apoptosis is induced in Jurkat cells treated with NF compounds, albeit to a lesser extent than that observed in treated HL 60 cells. Furthermore, NF compounds saturate the PBR at nanomolar concentrations (Campiani *et al.*, 1996), yet micromolar concentrations were required to induce apoptosis (Fig. 2).

In order to determine whether the induction of apoptosis involved the production of reactive oxygen intermediates, HL 60 cells were pretreated with a range of commonly used antioxidants including N-acetylcysteine, butylated hydroxyanisole, PDTC and the free radical scavenger, TEMPO, for 1 h prior to incubation with NF 225 for a further 8 h. Table 3 shows that these compounds do not protect against NF 225-induced apoptosis.

Because nuclear factor IB (NF kappa B) activation has previously been suggested to protect against apoptosis (Baichwal and Baeuerle, 1997), the effect of pretreating HL 60 cells with the cytokine, TNF-alpha, was determined. Pretreatment of HL 60 cells with TNF-alpha (10ng/ml), a concentration which was previously shown to activate NF kappa B (Baeuerle and Henkel, 1994), afforded no protection against NF 225-induced apoptosis (Fig. 5). To further determine the possible involvement of NF kappa B in the mechanism, the effect of the five most potent NF compounds in Hut 78 cells was then investigated. HUT 78 cells constitutively express NF kappa B (O'Connell *et al.*, 1995) which should protect them against apoptosis (Beg and Baltimore, 1996). However, the five NF compounds tested induced apoptosis in these cells with similar potency to that observed in the HL 60 cells (Fig. 6). The effect of NF 225 on NF kappa B activity (in HL 60 and HUT 78 cells) following induction of apoptosis was examined by band-shift analysis. Figs. 7 & 8 show that NF 225-induced apoptosis did not effect the basal levels of NF kappa B expression in HUT 78 and HL60 cells or the TNF-alpha-activated levels of NF kappa B in HL 60 cells.

The proenzyme form of caspase 3 (32 kDa) is cleaved by an

interleukin-1, β -converting enzyme (ICE)-like activity to produce the active form of the enzyme, which exists as a heterodimer of two subunits (p17 kDa & p12 kDa). The presence of procaspase 3 was detected in HL 60 and Hut 78 cells by Western blotting whole cell extracts with a monoclonal antibody against the 32 kDa proenzyme. NF 225-treatment of the cells activated caspase 3, as demonstrated by a disappearance of the proenzyme form in both HL 60 and Hut 78 cells (Fig. 9). When HL 60 cells were pretreated with z-DEVD-fmk, a specific inhibitor of caspase 3 (Nicholson & Thornbury, 1997), prior to incubation with either staurosporine (which has been reported to activate caspase 3 [Kluck et al., 1997]) or NF 225, no significant induction of apoptosis was detected (Fig. 10 & 11).

The apoptotic effect of a number of other compounds, namely NF 221, NF223, NF224, NF225 and NF325, NF212, NF299, NF297, 307 and 267 was examined in HL60 and Hut 78 cells. The results shown in Table 4 give the % apoptosis after treatment of the cells for 16 hours at a concentration of 25 μ M per relevant compound.

Similar experiments were carried out on the chronic myeloid leukemia (CML) cell lines Lama 84, K562 and KY0.1, which are generally accepted to be cells which are difficult to kill. Each cell line was treated with 10 μ M NF 225 for 20 hours and the percentage apoptosis measured. The results are shown in Table 5.

Discussion

The inventors have shown that the PBR ligands (PK 11195, Ro5-4864 and the NF compounds) induced apoptosis in the human cancerous cell lines, Jurkat, HL 60, Hut 78 and LAMA 84, K562 and KY0.1 cells. The characteristic morphological effects of apoptosis such as reduction in cell size, extensive membrane blebbing, condensation of chromatin and DNA fragmentation were observed. The NF compounds induced apoptosis with various potencies. While some of these compounds were found to have no effect, others e.g. NF 325, were found to be much more potent at inducing apoptosis than either PK 11195 or Ro5-4864.

In HL60 cells, induction of apoptosis by NF 225 was shown to be both dose- and time-dependent, the first sign of apoptosis being

observed after 4 hours. This apoptotic effect seems unrelated to a specific interaction of these drugs with the PBR as much higher concentrations of these ligands were required to induce apoptosis than were necessary to saturate the receptor. Furthermore, some of the NF compounds e.g NF 262, did not induce apoptosis, yet all of these drugs bind to the PBR with similar affinity (Campiani et al., 1996). In addition, certain NF compounds induce apoptosis in Jurkat cells, a cell line previously shown to lack the PBR (Carayon et al., 1996).

Several observations suggest an involvement of reactive oxygen species (ROS) in the signal transduction pathways leading to apoptosis (Jacobson, 1996). The addition of ROS or the depletion of endogenous antioxidants can induce apoptosis. This mode of cell death is sometimes associated with increases in intracellular ROS levels and the addition of exogenous antioxidants such as N-acetylcysteine (NAC) can inhibit apoptosis (see Buttke and Sandstrom, 1994 for review). However, in this work it has been shown that NF 225-induced apoptosis in HL 60 cells was unaffected by the presence of the antioxidants NAC and pyroiodithiocarbamate or by the presence of the spin trap and free radical scavenger, TEMPO. This would suggest that NF 225-induced apoptosis is not mediated by ROS. This is in agreement with recent reports (e.g. Jacobson, 1996) indicating that ROS are not necessarily a requirement for apoptosis.

Several recent papers (Beg and Baltimore, 1996; Wang et al., 1996; van Antwerp et al., 1996; Liu et al., 1996 and Wu et al., 1996) have shown that activation of the transcription factor NF kappa B is linked to apoptosis, with this factor playing an anti-apoptotic role, most likely by inducing expression of gene products that inhibit the apoptotic pathway. Inactivation of NF kappa B genetically or pharmacologically, appears to promote apoptosis (Baichwal and Baeuerle, 1997). Studies on "knock -out" mice that lack the 65 kDa RelA subunit of NF kappa B, as a result of targeted mutation of the Rel a gene, revealed that the mice die before birth and show massive degeneration of liver cells caused by apoptosis. However a general role for NF kappa B as a transcription factor that prevents apoptosis is far from established. A recent study with neurons suggested that NF kappa B can have the opposite effect. Grilli et al. (1996) showed that glutamate-induced toxicity in neuronal cells was found to be accompanied by the induction

of NF kappa B. In this case, NF kappa B activation may then cause cell death. In conclusion, the role of NF kappa B as a promoter or inhibitor of cell death may depend on both the cell type and the nature of the apoptosis-inducing stimulus (Baichwal and Baeuerle, 1997).

5

In the present study it was shown that while the NF compounds induced apoptosis, they did not effect either the basal levels of NF kappa B in HL 60 and HUT 78 cells, or TNF-alpha-activated levels of NF kappa B in HL 60 cells. Because HUT 78 cells constitutively express NF
10 kappa B, they are notoriously difficult to kill. However, in this work, the NF compounds induced apoptosis to a similar extent in both HL 60 and HUT 78 cells. It can thus be concluded that NF 225-induced apoptosis most likely employs an NF kappa B-independent mechanism. Furthermore this study argues against a general role for NF kappa B as a
15 transcription factor that prevents cell death.

Several anti-cancer drugs, such as the anthracycline antibiotic daunorubicin, activate NF kappa B in addition to inducing cell death. Daunorubicin is widely used in cancer chemotherapy and although its
20 mechanism of anti-tumour action is uncertain, it ultimately activates the event of apoptosis in cells. The concomitant activation of NF kappa B may counteract the therapeutic effects of daunorubicin and many other chemotherapeutic drugs. Therefore an anti-cancer drug therapy which does not activate NF kappa B may result in more effective anti-cancer
25 treatments.

Members of the caspase (ICE) family of cysteine proteases have been reported to play a pivotal role in the induction of apoptosis (Yuan *et al.*, 1993). Caspase activation is a widespread occurrence in
30 apoptosis and there are examples where caspase inhibition is sufficient (Beidler *et al.*, 1995; Sabbatini *et al.*, 1997) to inhibit cell death. It has been suggested, for several reasons, that of all the members of this family of enzymes, caspase 3 may play the most general role in mediating apoptosis in a variety of cells (Polverino and
35 Patterson, 1997). Firstly, caspase 3 is ubiquitously expressed in all tissues examined. Secondly, purified caspase 3 has been shown to cleave a variety of substrates, e.g. poly(ADP-ribose) polymerase (PARP) (Tewari *et al.*, 1995), that undergo cleavage during apoptosis. Finally, inhibition of caspase 3 blocks apoptosis in a variety of systems

(Nicholson *et al.*, 1995). This study demonstrates that induction of apoptosis by NF 225 is associated with activation of caspase 3.

Whether other components acting in parallel pathways are also
5 required, in addition to the activation of caspase 3, for the induction
of apoptosis by NF 225 has not been determined. The mechanism(s) of
activation of the caspase(s) and the potential targets involved in this
process will need to be identified. In conclusion, it has been
demonstrated that benzodiazepine-like compounds can induce apoptosis in
10 cancerous cell lines, indicating the potential of these compounds as
anti-tumour drugs.

SUMMARY

15 A range of Pyrrolobenzoxazepine derivatives (NF compounds) have
been shown to induce apoptosis, with various potencies, in the following
six human cancer cell lines: HL 60 (promyelocytic leukaemia), HUT 78 (T
cell Lymphoma), Jurkat (leukaemic T cell Lymphoblast) and LAMA 84, KYO.1
and K562 cells. The induction is both dose- and time-dependent. It does
20 not appear to involve the specific interaction of these ligands with the
peripheral-type benzodiazepine receptor (PBR), to which they bind with
high affinity, as much higher concentrations of these compounds were
required to induce apoptosis than were necessary to saturate the
receptor. In addition, some of the NF compounds induce apoptosis in
25 Jurkat cells, which have previously been reported to lack the PBR.

NF compound-induced apoptosis was unaffected by the presence of
antioxidants such as N-acetylcysteine, suggesting that the mechanism by
which these compounds cause apoptosis does not involve the production of
30 reactive oxygen intermediates.

Activation of the transcription factor NF kappa B has previously
been suggested to protect against apoptosis. Five selected NF compounds
induced apoptosis in HUT 78 cells (which contain constitutively active
35 NF kappa B), albeit with reduced potency to that observed in HL 60
cells. In addition, activation of NF Kappa B by the cytokine TNF-alpha
in HL 60 cells prior to the addition of NF 225 did not protect these
cells against apoptosis. Furthermore, NF 225-induced apoptosis did not
effect the levels of NF kappa B activity in either HUT 78 or HL 60

cells. These results would suggest that NF 225-induced apoptosis employs an NF kappa B-independent mechanism.

Caspases are a closely related family of cysteine proteases that
5 play a key role in apoptosis. In this study it was shown that caspase 3
becomes activated (as monitored by the disappearance of the inactive
form of the enzyme) during NF 225-induced apoptosis. In addition,
pretreatment of HL 60 cells with a specific inhibitor of caspase 3,
z-DEVD-fmk, inhibits apoptosis induced by NF 225. These results indicate
10 that caspase 3 plays an important role in apoptosis in response to NF
225.

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Table 1.1 Summary of peripheral-type and central-type benzodiazepine receptor characteristics

	Peripheral-type	Central-type
Tissue distribution	Ubiquitously distributed in peripheral tissues (especially steroidogenic) as well as in glia of CNS	Neuronal
Subcellular localisation	Mitochondrial outer membrane	Plasma membranes
Synthetic ligands	Isoquinolines (e.g. PK 11195), benzodiazepines-(e.g. Ro5-4864, species-dependent, GABA-insensitive)	Benzodiazepines-(e.g. clonazepam, GABA-sensitive)
Endogenous ligands	diazepam binding inhibitor (DBI)	DBI
Molecular components	Isoquinoline binding protein(18kDa), mitochondrial VDAC(32kDa), ANC(30kDa)	Heterogenous α , γ subunits of GABA _A receptor
Effector mechanism	cholesterol transport?	Regulates chloride flux by modulating GABA binding to GABA _A receptor

TABLE 2. Effect of PBR ligands on apoptosis in HL 60 cells.

5	Drug [50 μ M]	% Apoptosis	% Necrosis
	Control (1% EtOH)	1.1 \pm 1.2	1.5 \pm 2.0
	Diazepam	1.7 \pm 1.2	0.6 \pm 1.0
	Clonazepam	1.2 \pm 0.2	0.9 \pm 0.1
10	Ro5-4864	8.7 \pm 1.1	1 \pm 0.2
	PK 11195	7.1 \pm 1.0	0.3 \pm 0.2
	NF 149	5.8 \pm 1.0	2.2 \pm 0.3
15	NF 182	3.8 \pm 1.8	1.7 \pm 1.0
	NF 198	5.1 \pm 2.0	0.3 \pm 0.2
	NF 212	11.8 \pm 3.0	0.9 \pm 0.7
	NF 213	6.3 \pm 2.0	1.2 \pm 0.4
20	NF 221	52.6 \pm 1.2	5.3 \pm 2.3
	NF 223	71.3 \pm 2.7	8.5 \pm 1.5
	NF 224	55.7 \pm 3.2	3.2 \pm 1.2
	NF 225	60.4 \pm 2.1	4.5 \pm 1.5
25	NF 262	2.9 \pm 1.0	0
	NF 267	43.7 \pm 2.3	5.6 \pm 1.3
	NF 325	67.4 \pm 3.2	5.7 \pm 2.3

30 HL 60 cells were seeded at a density of 3×10^5 cells/ml into 24-well plates (1ml/well) and treated, for 16 h, with the various drugs (50 μ M) listed above. Control wells contained 1% (vol/vol) ethanol. Percent apoptosis and necrosis was determined after cytopinning and staining the cells. The values represent the mean \pm range for two experiments.

35

TABLE 3 Lack of protective effect of antioxidant compounds on NF 225-induced apoptosis in HL 60 cells

5	Pretreatment	Concentration
	NAC	5mM
	PDTC	100 μ M
10	BHA	400 μ M
	α -Lipoic acid	4mM
	TEMPO	1 μ M

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Table 4

	Compound	Experimental formula	Molecular weight	% Apoptosis	
				HL-60	HUT 78
5	NF212,	C21 H17 N03,	331.4,	11.8%	16.4%
	NF299,	C20 H16 N2 O3,	332.4,	13.5%	15.2%
	NF297,	C22 H21 N3 O3,	375.4,	11.4%	15.4%
10	NF307,	C24 H23 N3 O3,	401.5,	4.6%	13.6%
	NF267,	C21 H18 N2 O3,	346.4,	15.6%	9.0%.

15 **Table 5**

	Cell Line	% Apoptosis
20	Lama 84	34 \pm 2
	K562	57 \pm 3
	KY0.1	55 \pm 4

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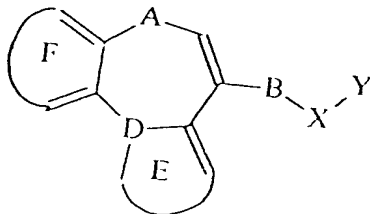
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CLAIMS

1. A pharmaceutical composition comprising an apoptosis-inducing amount of a compound having the general formula:-



10 wherein R_1 represents

unsubstituted straight chain, or branched C_1 - C_{10} alkyl or unsubstituted C_3 - C_{10} cycloalkyl,

or straight chain or branched C_1 - C_{10} alkyl substituted with one or more substituents or C_3 - C_{10} cycloalkyl substituted with one or more substituents,

unsubstituted straight chain, or branched C_2 - C_{10} alkylene or unsubstituted C_3 - C_{10} cycloalkylene,

or straight chain or branched C_2 - C_{10} alkylene substituted with one or more substituents or C_2 - C_{10} cycloalkylene substituted with one or more substituents,

an unsubstituted phenyl group or phenyl substituted by one or more substituents,

25 an unsubstituted C_6 - C_{20} aryl group or a C_2 - C_{20} aryl group substituted with one or more substituents

unsubstituted naphthyl substituted with one or more substituents, preferably the substituted or unsubstituted naphthyl being a 1- or 2-naphthyl,

30 an unsubstituted biphenyl or a biphenyl substituted with one or more substituents, preferably the substituted or unsubstituted biphenyl being a 4- biphenyl,

35 an unsubstituted five or six numbered heterocyclic group with at least one hetero atom and wherein the or each heteroatom is selected from N, O, S or C, five or six numbered heterocyclic group with at least one heteroatom and wherein the or each heteroatom is selected from

N,O,S, the heterocyclic group being substituted with one or more substituents preferably the heterocyclic being selected from 2- and 3-pyridine, pyrrole, thiophene;

5 where A represents N,O,S or the group CH₂; and

 where B represents N,O, or the group CH₂; and

 where D represents N or the group CH₂

10

wherein the cyclic group labelled E is taken together with D to form a pyrrole or indole ring which may be substituted;

and wherein the cyclic group labelled F represents:

15

an unsubstituted phenyl group or phenyl substituted by one or more substituents,

an unsubstituted C₆-C₂₀ aryl group or a C₂-C₂₀ aryl group substituted with one or more substituents

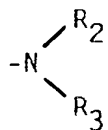
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unsubstituted naphthyl substituted with one or more substituents, or a 5 or 6 membered heterocyclic group, with at least one heteroatom and wherein the or each heteroatom is selected from O,N or S, and a 5 or 6 membered heterocyclic group with at least one heteroatom and where the or each heteroatom is selected from O,N or S substituted with one or more substituents, and preferably when the heterocycle is pyridine or thiophene wherein X represents the group X=O, C=S or CH₂; and

25

where Y represents the group

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35 wherein R₂ and R₃ are independently hydrogen, or

unsubstituted straight chain, or branched C₁-C₁₀ alkyl or unsubstituted C₃-C₁₀ cycloalkyl,

or straight chain or branched C₁-C₁₀ alkyl substituted with one or more substituents or C₃-C₁₀ cycloalkyl substituted with one

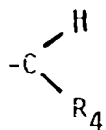
or more substituents,

unsubstituted straight chain, or branched C_2-C_{10} alkylene or unsubstituted C_3-C_{10} cycloalkylene,

or straight chain or branched C_2-C_{10} alkylene substituted with
 5 one or more substituents or C_2-C_{10} cycloalkylene substituted with one or more substituents,

or Y represents the group

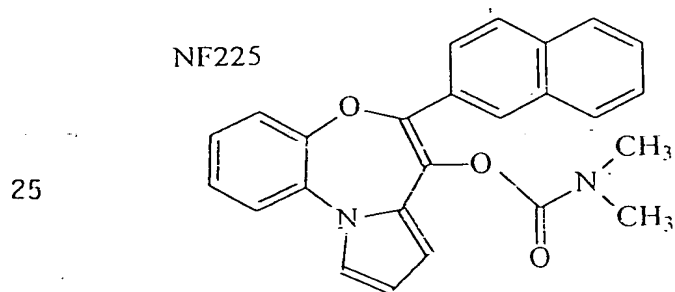
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wherein R_4 is $(CH_2)_n CH_3$ wherein N is 0 or an integer from 1 to
 15 12 and may be substituted.

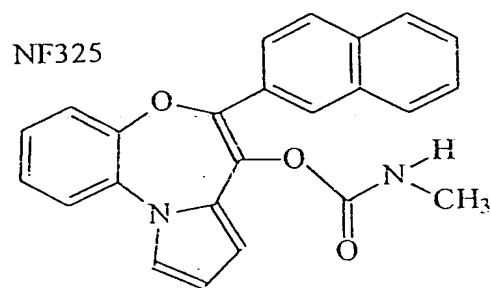
2. A composition as claimed in claim 1 in which R_1 or F represent a naphthyl group.

3. A composition as claimed in claim 1 wherein the compound is
 20 selected from those having the formulae:-



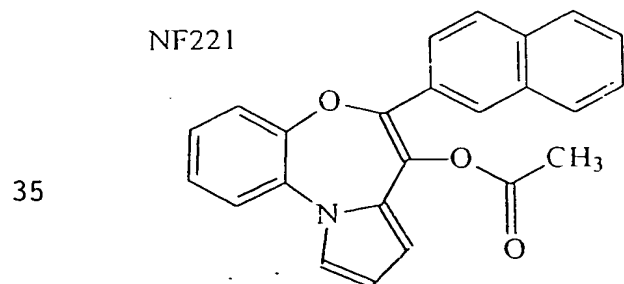
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 MW396.4

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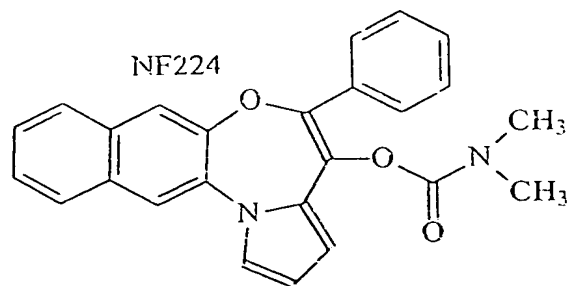
$C_{24}H_{18}N_2O_3$
 MW382.4

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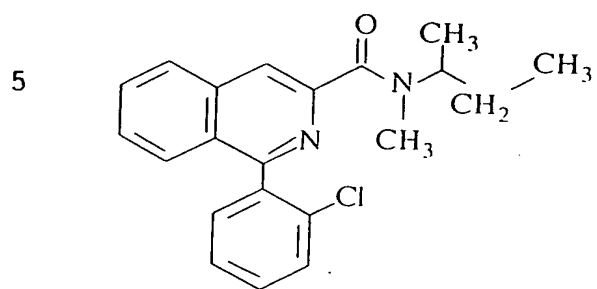


$C_{24}H_{17}NO_3$
 MW367.4

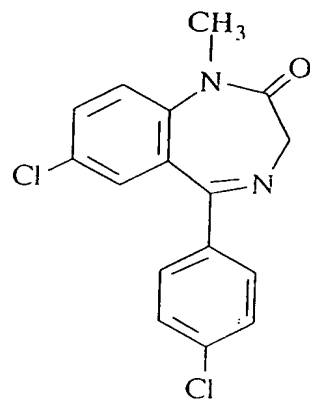
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$C_{25}H_{20}N_2O_3$
 MW396.4

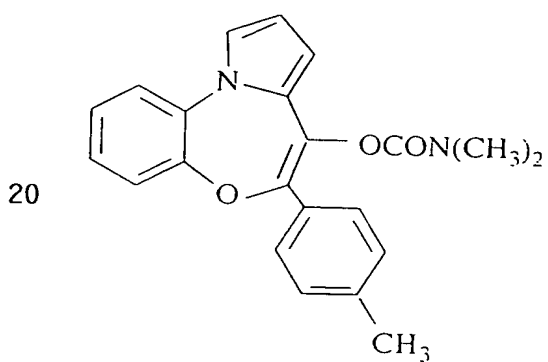


PK 11195

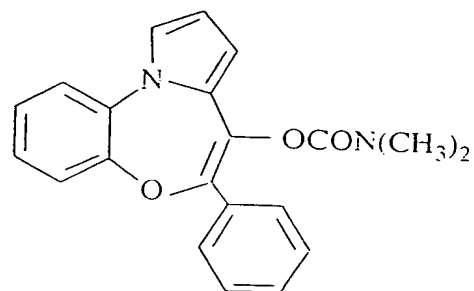


Ro5-4864

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NF 213.

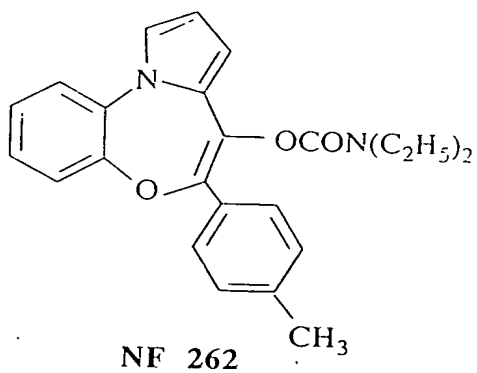


NF 182

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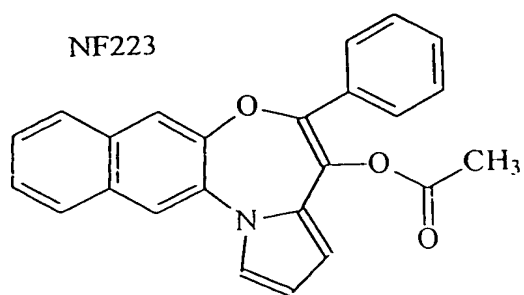
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NF 262

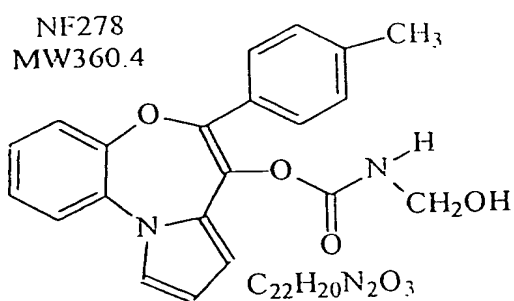
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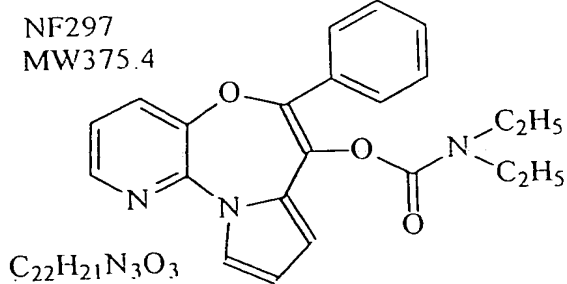
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$C_{24}H_{17}NO_3$ MW367.4

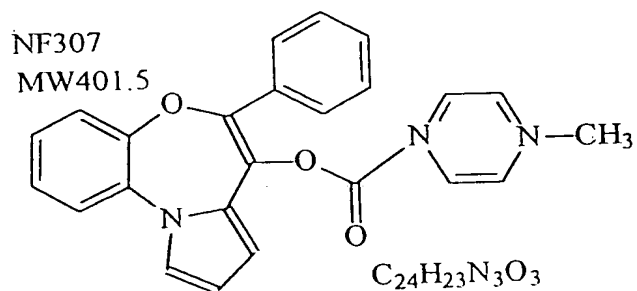
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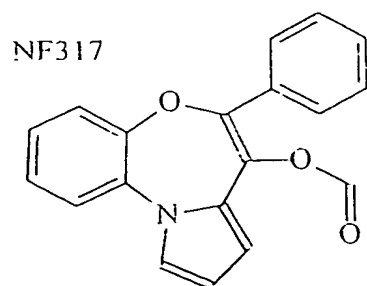
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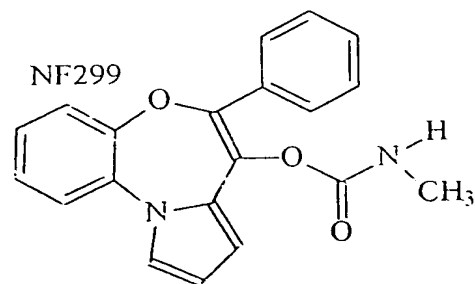


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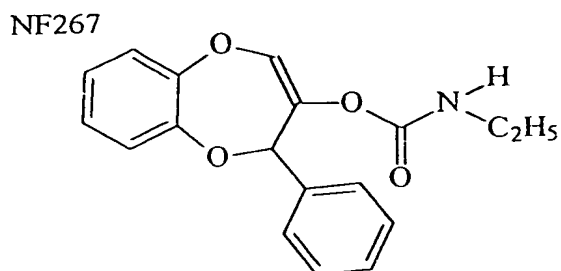


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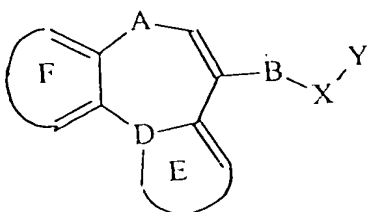
$C_{22}H_{18}N_2O_3$
MW358.4



$C_{20}H_{16}N_2O_3$
MW332.4



4. Use of a compound having the general formula



wherein R_1 represents

unsubstituted straight chain, or branched C_1 - C_{10} alkyl or unsubstituted C_3 - C_{10} cycloalkyl,

or straight chain or branched C_1 - C_{10} alkyl substituted with one or more substituents or C_3 - C_{10} cycloalkyl substituted with one or more substituents,

unsubstituted straight chain, or branched C_2 - C_{10} alkylene or unsubstituted C_3 - C_{10} cycloalkylene,

or straight chain or branched C_2 - C_{10} alkylene substituted with one or more substituents or C_2 - C_{10} cycloalkylene substituted with one or more substituents,

an unsubstituted phenyl group or phenyl substituted by one or more substituents,

an unsubstituted C_6 - C_{20} aryl group or a C_2 - C_{20} aryl group substituted with one or more substituents

unsubstituted naphthyl substituted with one or more substituents, preferably the substituted or unsubstituted naphthyl being a 1- or 2-naphthyl,

an unsubstituted biphenyl or a biphenyl substituted with one or more substituents, preferably the substituted or unsubstituted biphenyl being a 4- biphenyl,

an unsubstituted five or six numbered heterocyclic group with at least one hetero atom and wherein the or each heteroatom is selected from N,O,S or C, five or six numbered heterocyclic group with at least one heteroatom and wherein the or each heteroatom is selected from

- 5 N,O,S, the heterocyclic group being substituted with one or more substituents preferably the heterocyclic being selected from 2- and 3-pyridine, pyrrole, thiophene;

10 where A represents N,O,S or the group CH₂; and

where B represents N,O, or the group CH₂; and

where D represents N or the group CH₂

- 15 wherein the cyclic group labelled E is taken together with D to form a pyrrole or indole ring which may be substituted;

and wherein the cyclic group labelled F represents:

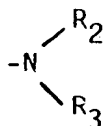
- 20 an unsubstituted phenyl group or phenyl substituted by one or more substituents,

an unsubstituted C₆-C₂₀ aryl group or a C₂-C₂₀ aryl group substituted with one or more substituents

- 25 unsubstituted naphthyl substituted with one or more substituents, or a 5 or 6 membered heterocyclic group, with at least one heteroatom and wherein the or each heteroatom is selected from O,N or S, and a 5 or 6 membered heterocyclic group with at least one heteroatom and where the or each heteroatom is selected from O,N or S substituted with one or
30 more substituents, and preferably when the heterocycle is pyridine or thiophene wherein X represents the group X=O, C=S or CH₂; and

where Y represents the group

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wherein R₂ and R₃ are independently hydrogen, or

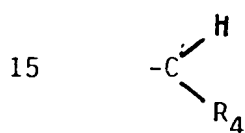
unsubstituted straight chain, or branched C_1 - C_{10} alkyl or unsubstituted C_3 - C_{10} cycloalkyl,

or straight chain or branched C_1 - C_{10} alkyl substituted with one or more substituents or C_3 - C_{10} cycloalkyl substituted with one or more substituents,

unsubstituted straight chain, or branched C_2 - C_{10} alkylene or unsubstituted C_3 - C_{10} cycloalkylene,

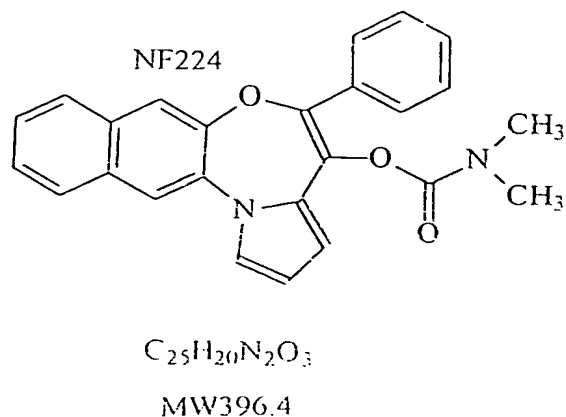
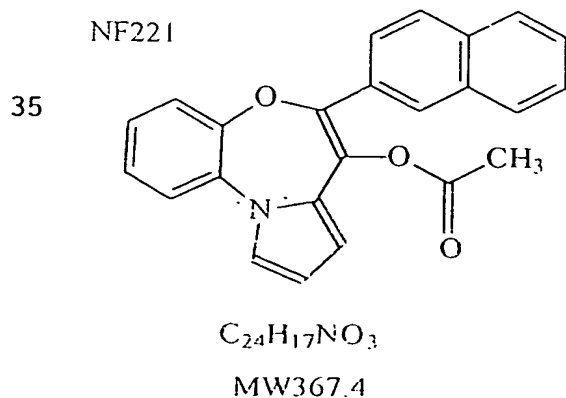
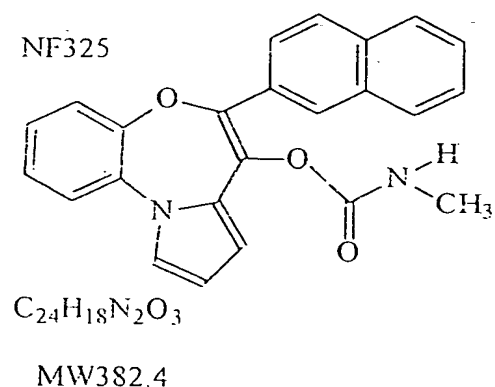
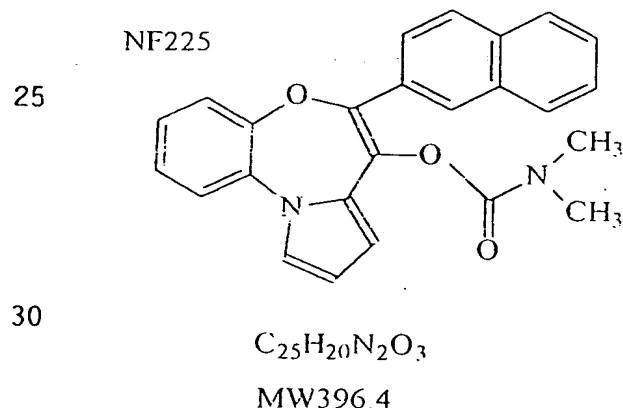
or straight chain or branched C_2 - C_{10} alkylene substituted with one or more substituents or C_2 - C_{10} cycloalkylene substituted with one or more substituents,

or Y represents the group

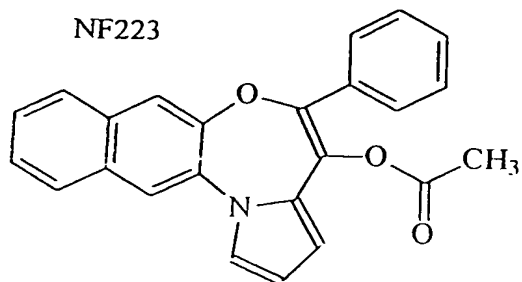


wherein R_4 is $(\text{CH}_2)_n \text{CH}_3$ wherein N is 0 or an integer from 1 to 12 in the preparation of a medicament for the treatment of tumours or other cancerous conditions.

5. Use of a compound selected from those having the formula:

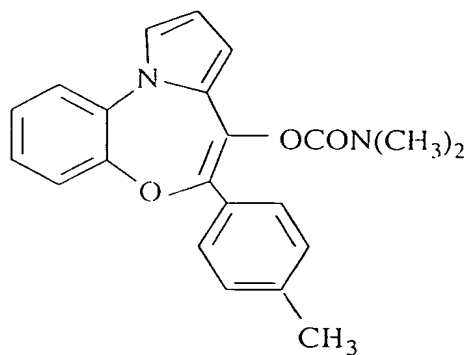


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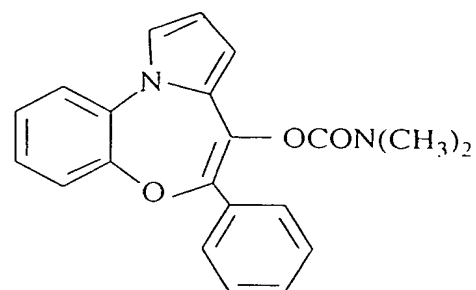


$C_{24}H_{17}NO_3$ MW367.4

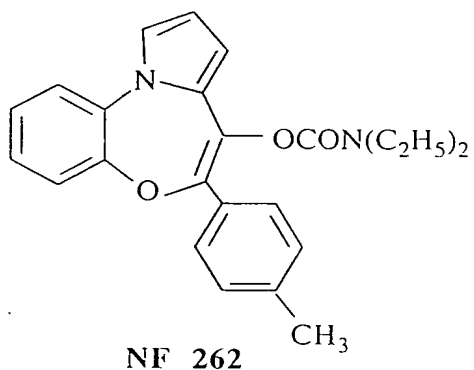
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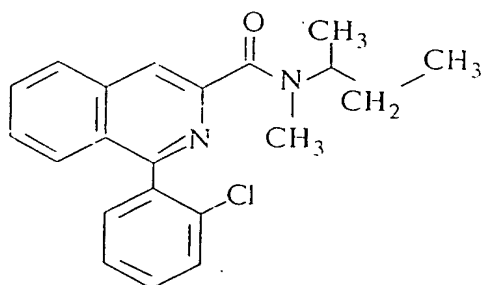


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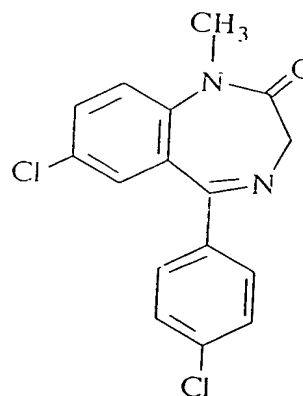


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in the preparation of a medicament for the treatment of tumours or other cancerous conditions.

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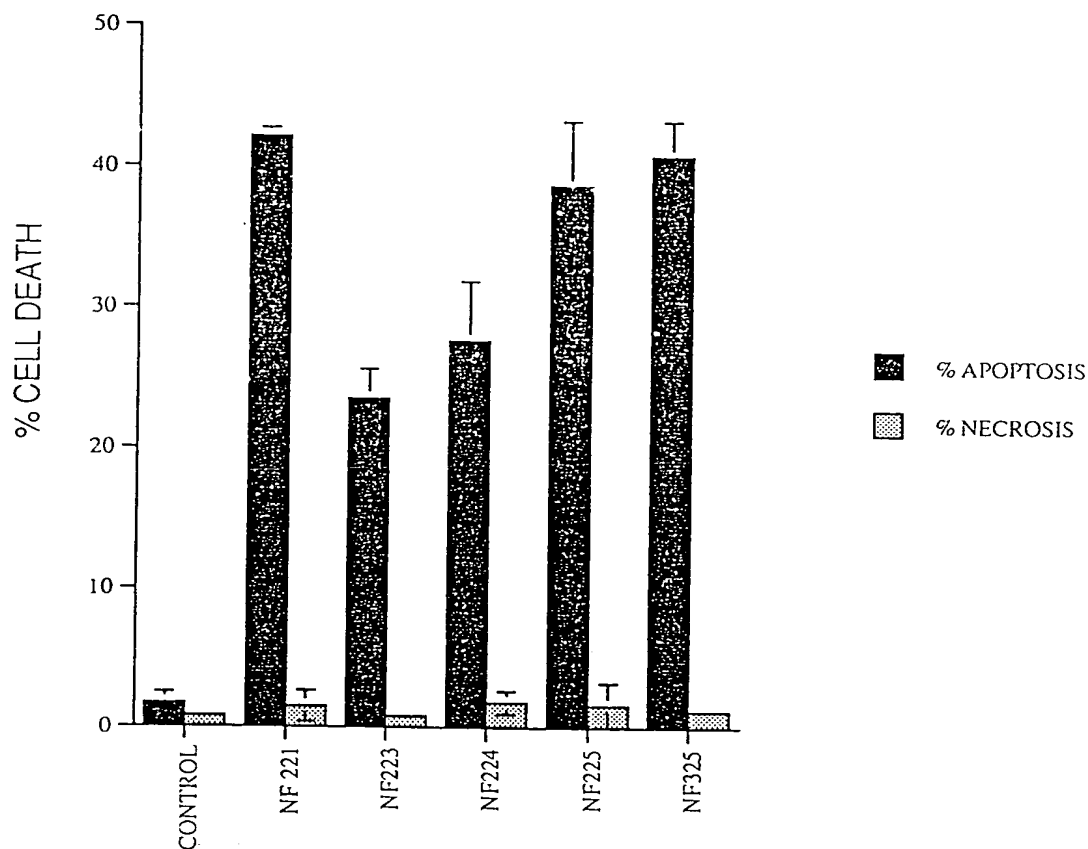


FIG. 1 Effect of selected NF compounds on apoptosis in HL 60 cells.

HL 60 cells, seeded at the appropriate density into 24-well plates (1ml/well), were incubated with one of the following drugs: NF 221,223,224,225 or 325 (each at a final concentration of 10 μ M). The control well contained ethanol at a final concentration of 1% (vol/vol). After 16 h, the percent apoptosis and necrosis was determined after cytospinning and staining the cells using the RAPIDIFF kit. Values represent the means \pm SEM for three experiments.

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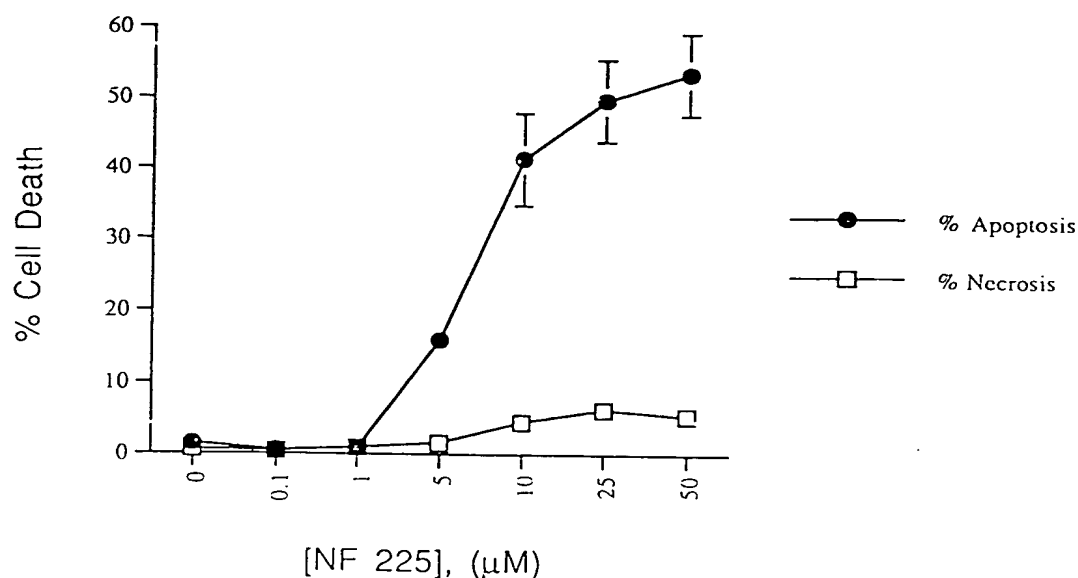


FIG. 2 Dose-response of NF 225-induced apoptosis in HL 60 cells. HL 60 cells were seeded at a density of 3×10^5 cells/ml into 24-well plates (1ml/well) and were treated with NF 225 over a range of concentrations (0-50μM). After 16 h, the percentage apoptosis (●) and necrosis (□) were determined as described in Materials and Methods. Points shown are the means \pm SEM for three experiments.

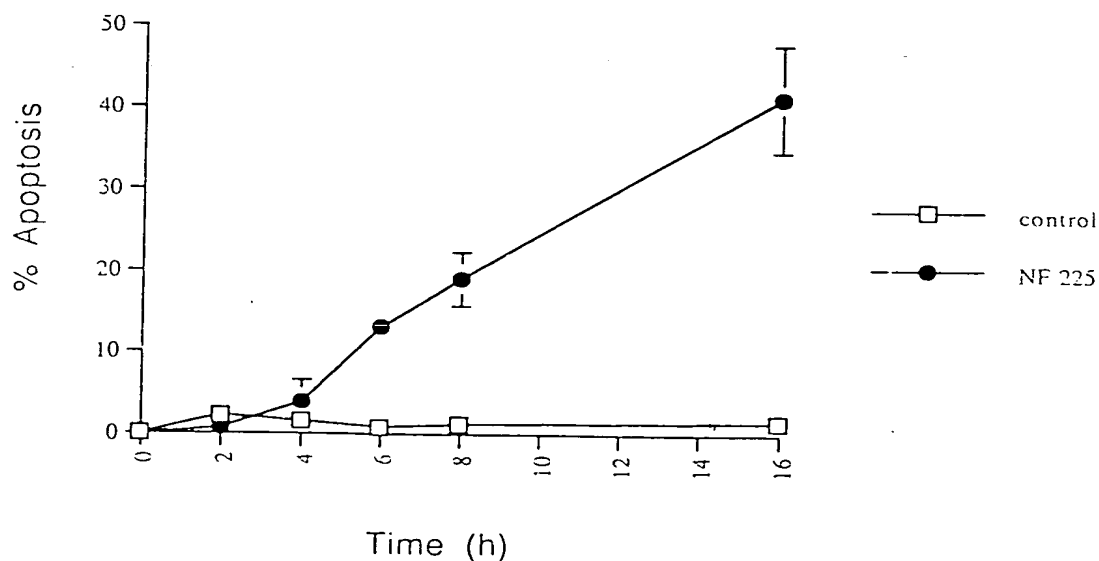


FIG. 3 Time-course of NF 225-induced apoptosis in HL-60 cells. HL-60 cells were seeded at the appropriate density into 24-well plates (1ml/well) and treated with NF 225 (10μM). Samples were taken at 2,4,6,8 and 16 h and the percent apoptosis and necrosis for the control(□) and NF 225-treated (●) cells determined, as described in Materials and Methods. Points shown are the means \pm SEM for three experiments.

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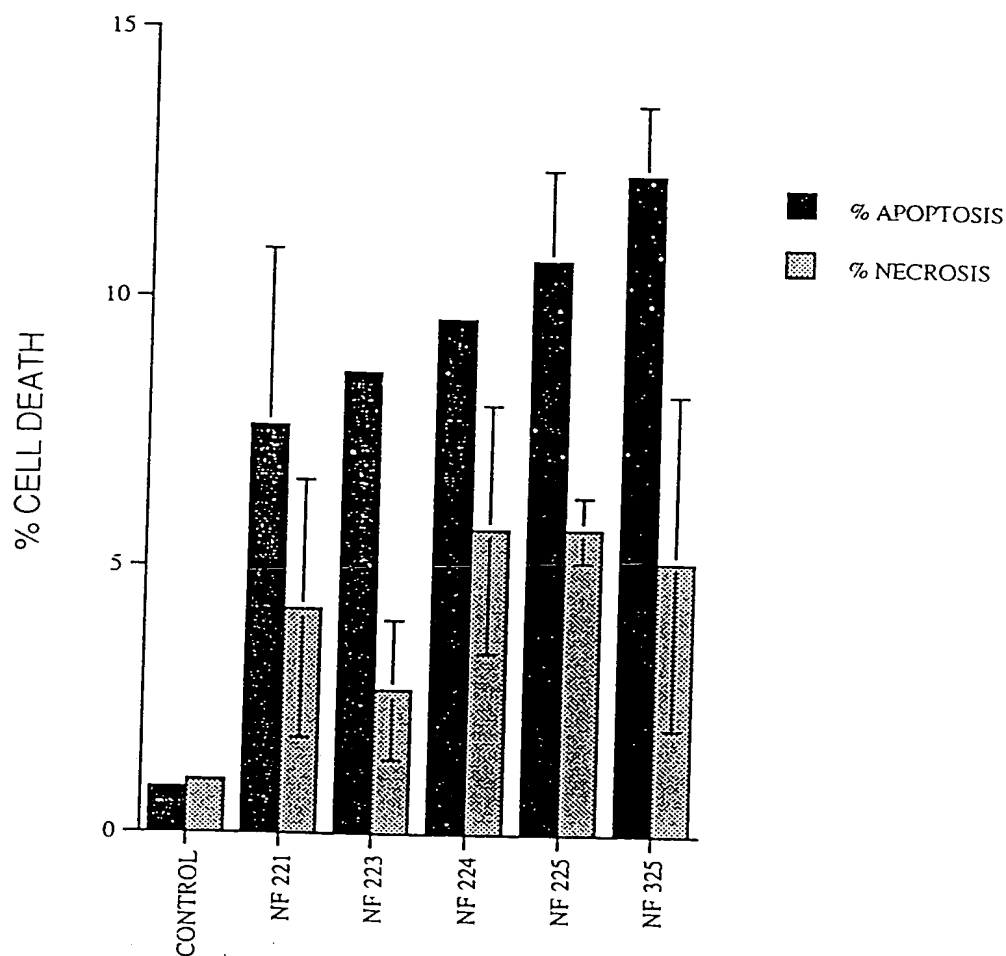


FIG. 4 Effect of NF compounds on apoptosis in Jurkat cells
Jurkat cells, seeded at a density of 3×10^5 cells/ml into 24-well plates (1ml/well), were incubated for 16 h with one of the following NF compounds (at a final concentration of $10 \mu\text{M}$): 221, 223, 224, 225 or 325. The control well contained a final concentration of 1% (vol/vol) ethanol. Percent apoptosis and necrosis were determined as described in Materials and Methods. The values represent the means \pm range for two experiments.

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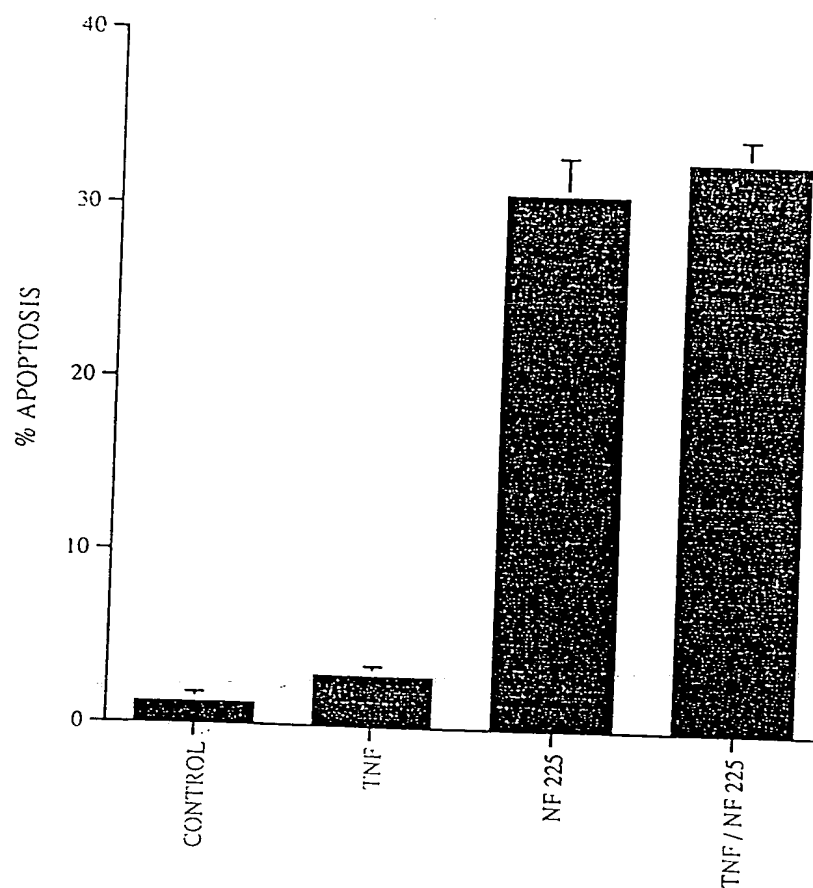


FIG. 5 Effect of TNF- α on NF 225-induced apoptosis in HL 60 cells. HL 60 cells were seeded at the appropriate density into 24-well plates (1ml/well), pretreated with TNF (10ng/ml) for 24 h and then incubated with NF 225 (10 μ M) for a further 16 h. The control well contained ethanol at a final concentration of 1% vol/vol. Percent apoptosis and necrosis were determined after cytopinning and staining the cells. The values represent the means \pm range for two experiments.

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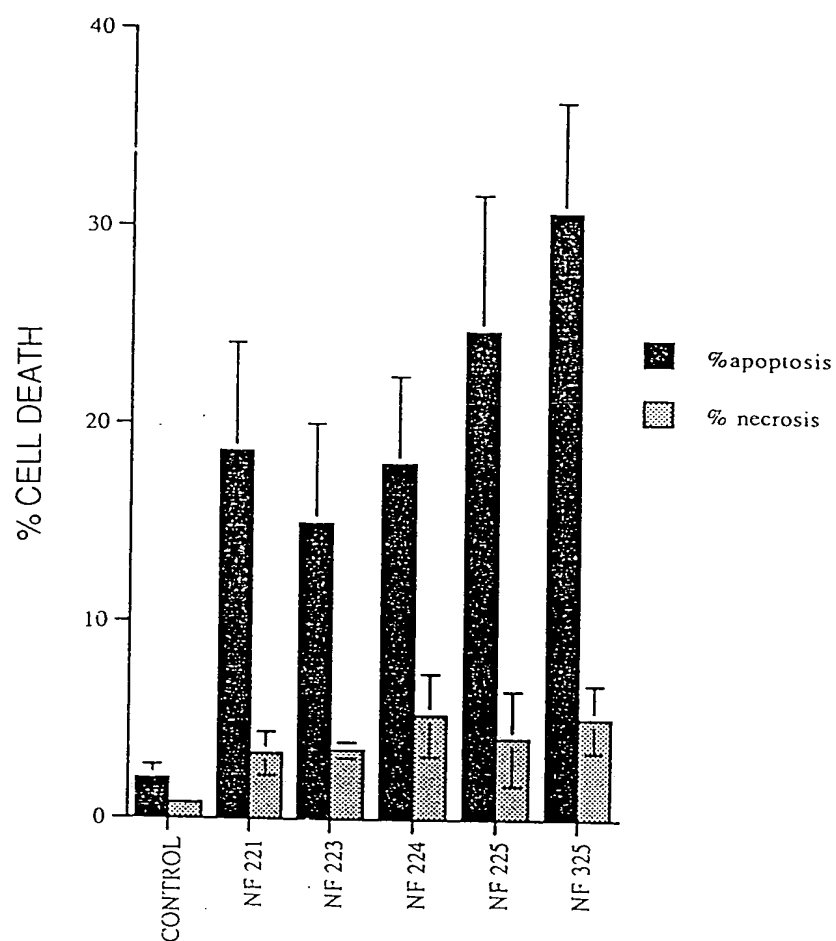


FIG. 6 Effect of selected NF compounds on apoptosis in HUT 78 cells
HUT 78 cells were seeded at the appropriate density into 24-well plates (1ml/well) and were incubated with one of the following drugs NF 221, 223, 224, 225 or 325 (at a final concentration of 10 μ M). The percent apoptosis and necrosis was determined as described in the Materials and Methods. The values represent the means \pm SEM for three experiments.

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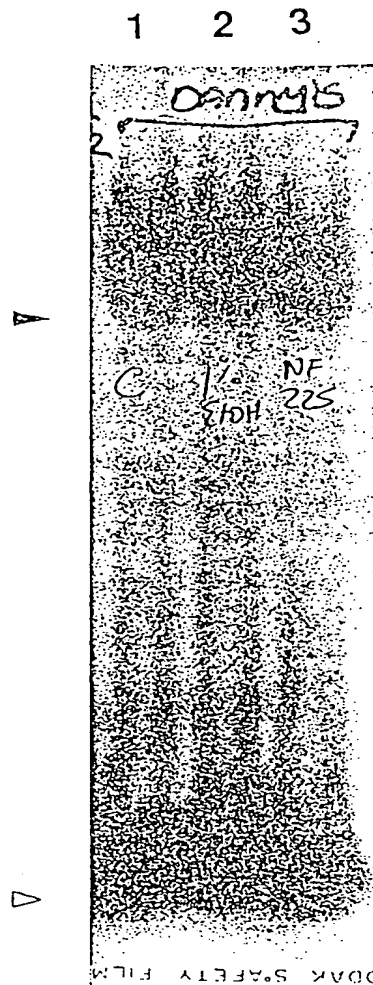


FIG. 7 Band-shift analysis of the effect of NF 225 on NF κ B activity in HUT 78 cells

HUT 78 cells were seeded at a density of 3×10^5 cells/ml into 24-well plates (1ml/well) and were incubated with NF 225 at a final concentration of $10 \mu\text{M}$ (lane 3) or in the presence or absence of 1% vol/vol ethanol (lanes 1&2 respectively), for 16 h. Nuclear extracts were prepared and assayed for NF κ B binding activity as described in the Materials and Methods. The filled arrowhead (▲) indicates induced DNA-protein complexes and the open arrowhead (△) indicates unbound DNA fragments.

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FIG. 8 Band-shift analysis of the effect of NF 225 on NF κ B activity in TNF- α -pretreated HL60 cells. HL 60 cells were seeded at the appropriate density, pretreated in the presence (lane 4) or absence (lane 3) of TNF- α (10ng/ml) for 1 h prior to incubation for a further 16 h with NF 225 (10 μ M). Control cells were incubated in ethanol at a final concentration of 1% (vol/vol) in the presence (lane 2) or absence of TNF- α (lane 1). Nuclear extracts were prepared and assayed for NF κ B binding activity as described in the Materials and Methods. The filled arrowhead () indicates induced DNA-protein complexes and the open arrowhead () indicates unbound DNA fragments.

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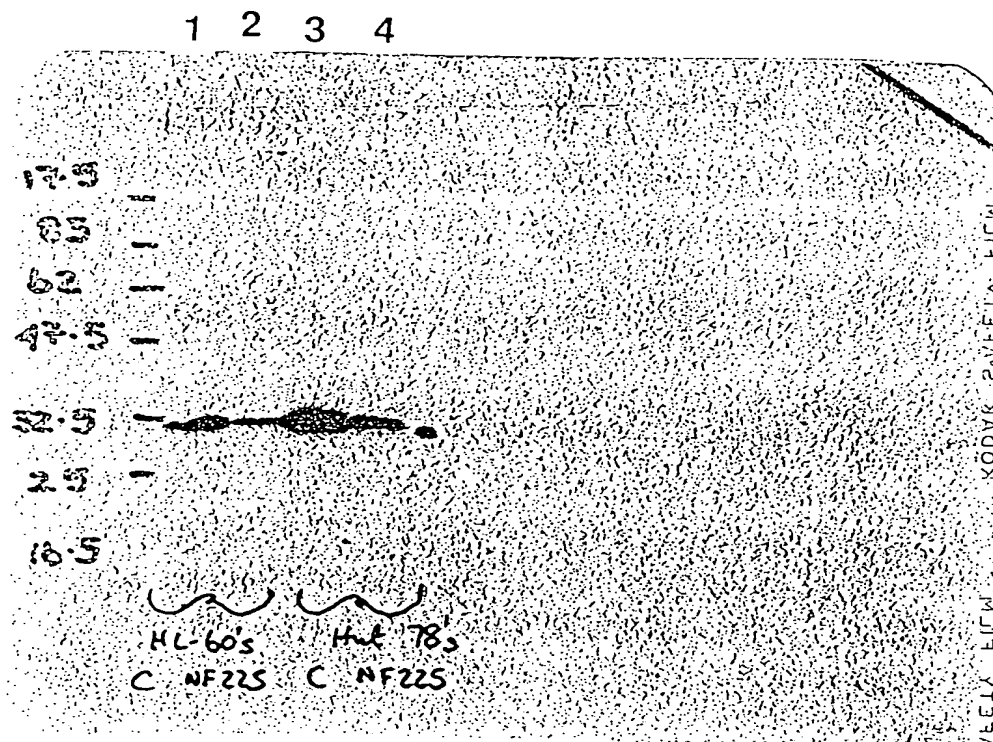


FIG. 9 Western blot analysis of the proenzyme form of caspase 3 in NF 225-treated cells. Hut 78 or HL 60 cells were seeded at the appropriate density and treated with NF 225(10 μ M) for 16 h. Samples (20 μ g) were electrophoresed using a 15% (wt/vol) polyacrylamide gel containing 0.01% (vol/vol) SDS, transferred onto nitrocellulose and blotted with anti-procaspase 3 (1: 1000 dilution). Lanes 1 and 2 contain control and treated HL 60 cells respectively. Lanes 3 and 4 contain control and NF 225-treated HUT 78 cells respectively.

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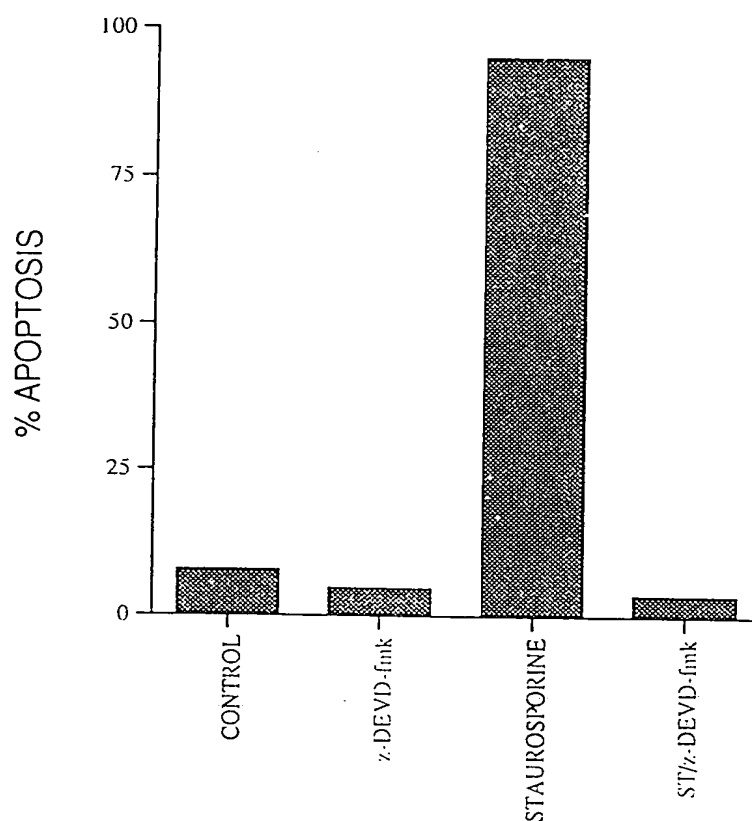


FIG. 10 Effect of z-DEVD-fmk on staurosporine-induced apoptosis in HL 60 cells.

HL 60 cells, seeded at the appropriate density into 24-well plates (1ml/well), were pretreated with z-DEVD-fmk (200 μ M) for 1 h prior to treatment with staurosporine (1 μ M) for 8 h. All wells (including controls) contained ethanol and DMSO at a final concentration of 1% (vol/vol) and 0.05% (vol/vol) respectively. Values were determined from a single experiment.

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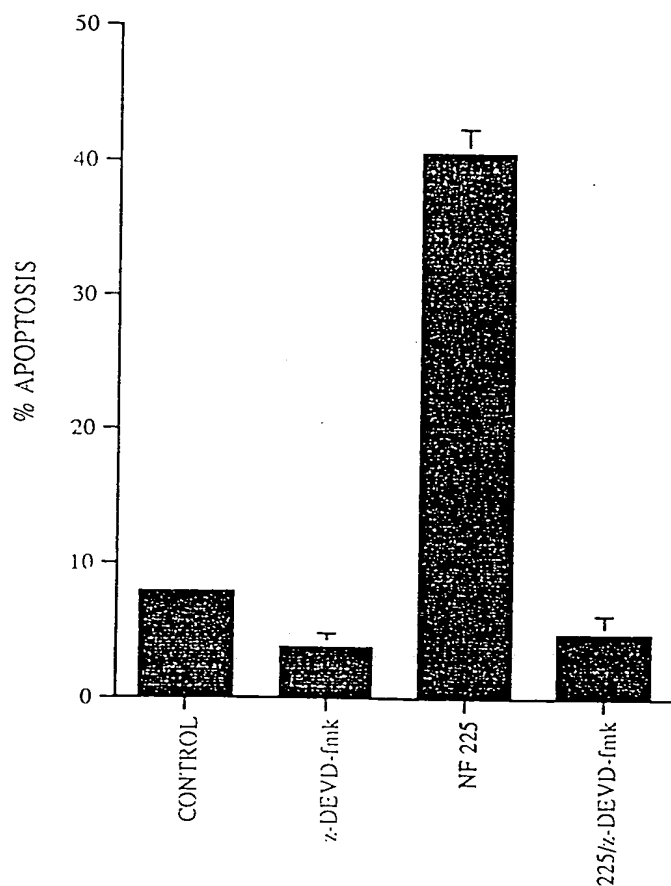


FIG. 11 Effect of z-DEVD-fmk on NF 225-induced apoptosis in HL 60 cells. HL 60 cells, seeded at the appropriate density into 24-well plates (1ml/well), were pretreated with z-DEVD-fmk (200 μ M) for 1 h prior to treatment with NF 225 (10 μ M) for 8 h. All wells (including controls) contained ethanol and dimethylsulphoxide (DMSO) at a final concentration of 1% (vol/vol) and 0.05% (vol/vol) respectively. Values represent the means \pm SEM for two experiments.



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